Remarks

Claims 1 and 9-17 have been canceled without prejudice or disclaimer. Applicants reserve the right to pursue the subject matter encompassed by all canceled claim in one or more divisional or continuation applications. Claims 99-101 have been amended to correct an minor clerical error in claim dependency. Claims 125 and 126 have been added to expand the embodiments of the elected subject matter. Support for new claims 125 and 126 can be found in the specification as filed. For example, support can be found in the specification at page 6, first paragraph. Upon entry of the present amendments, claims 21-24, 26-49, 51-76, 78-101 and 103-126 will be pending. Claims 21-24, 26-37, 46-49, 51-63, 72-76, 78-89, 98-101, 103-115 and 124-126 are under consideration. Claims 38-45, 64-71, 90-97, and 116-123 are presently withdrawn from consideration pending rejoinder. *See*, Provisional Election With Traverse, page 3, filed December 3, 2001. No new matter has been added.

In the Advisory Action, an enablement rejection of the currently pending claims was maintained. *See*, Paper No. 20, pages 1-5. In particular, claims 21-24, 26-37, 46-49, 51-63, 72-76, 78-89, 98-101, 103-115 and 124 were rejected as allegedly not enabled by the specification because "no one would believe it more likely than not that the presently claimed antibodies are useful for detecting or targeting colon cancer cells..." *See*, Paper No. 20, page 3 last line to page 4, second line.

Applicants respectfully disagree and herewith submit third party evidence in support of Applicants' position. In particular, Applicants submit Exhibit A (Violette, et al., "Reg IV, A New Member of the Regenerating Gene Family is Overexpressed in Colorectal Carcinomas", *Int. J. Cancer*, 103:185-193 (2003)).[1] The authors of the publication in Exhibit A analyzed Reg IV mRNA expression via Northern blot, differential display PCR, real time-PCR, and *in situ* hybridization assays. From these assays the authors concluded that Reg IV is overexpressed in colorectal carcinoma. As one example, the authors found that northern blot analysis and real-time PCR showed Reg IV expression at significantly higher levels in 71% of colorectal tumors compared to normal colon. *See*, Exhibit A at page 185 (abstract); page 188, right column, last paragraph to page 189, right column, penultimate paragraph. In summary, the authors concluded:

[1] The Reg IV and Colon Specific protein sequences are 100% identical (*i.e.*, Reg IV ORFs encoded by GenBank Accessions AF345934 and AY007243 are 100% identical to SEQ ID NO:2 of the present application).

> Our data concerning RegIV expression, as assessed by studying the mRNA level are in accordance with the suggestion of Macadam and collaborators[2] that colorectal tumor cells expressing REG proteins have an advantage in terms of survival. Our results suggest that Reg IV-positive cells in colorectal tumors may also have a selective advantage during drug treatment.

See, Exhibit A, page 192, right column, last paragraph (emphasis added). Hence, this publication demonstrates that in view of mRNA expression data alone, those of ordinary skill in the art would "believe it more likely than not that the presently claimed antibodies are useful for detecting or targeting colon cancer cells." Therefore, in consideration of Exhibit A and the previous explanations, data, and Declaration submitted, Applicants respectfully request that the rejection of claims 21-24, 26-37, 46-49, 51-63, 72-76, 78-89, 98-102, 103-115 and 124 under 35 U.S.C. § 112 be withdrawn.

Should the Examiner disagree with the present reply, Applicants respectfully request a personal interview with the Examiner prior to further examination of the instant Continued Prosecution Application (CPA). *See, e.g.*, M.P.E.P. § 706.07(b). The Examiner is invited to call the undersigned at the phone number provided below to schedule the requested interview.

No fee, beyond that which has been submitted, is believed due with this reply. However, should the Patent Office determine otherwise, please charge the required fee to Human Genome Sciences, Inc., deposit account no. 08-3425.

Dated: 11 April 2003

Respectfully submitted,

By _____
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[2] Macadam, et al., "Death from early colorectal cancer is predicted by the presence of transcripts of the REG gene family," Br. J. Cancer, 88:188-195 (2000).

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Soppet et al

Application No.: 09/525,041 Group Art Unit: 1642

Filed: March 14, 2000 Examiner: S. Ungar

For: Colon Specific Gene and Protein

VERSION WITH MARKINGS TO SHOW CHANGES MADE

The application has been amended as follows.

In the Claims:

Claims 1 and 9-17 have been canceled without prejudice or disclaimer.

Previous claims 99-101 have been amended as follows:

99. The antibody or portion thereof of claim 98 ~~97~~ produced by immunizing an animal with protein (a).

100. The antibody or portion thereof of claim 98 ~~97~~ produced by immunizing an animal with protein (b).

101. The antibody or portion thereof of claim 98 ~~97~~ produced by immunizing an animal with protein (c).

New claims 125 and 126 have been added:

125. (New) The antibody of claim 21, wherein said antibody binds the lectin domain of SEQ ID NO:2.

126. (New) The antibody of claim 73, wherein said antibody binds the lectin domain of the polypeptide encoded by the cDNA in ATCC Deposit Number 97129.

Int. J. Cancer: 103, 185–193 (2003)

Publication of the International Union Against Cancer

REG IV, A NEW MEMBER OF THE REGENERATING GENE FAMILY, IS OVEREXPRESSED IN COLORECTAL CARCINOMAS

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A better understanding of the mechanisms by which colon tumor cells are able to survive exposure to drugs would be valuable for the development of new therapeutic strategies. We used differential display-PCR to compare gene expression in the drug-sensitive HT-29 colon cancer cell line and 3 drug-resistant subpopulations derived from this parental cell line. One of the genes identified is a new gene, *Regenerating IV gene* (*Reg IV*), and was strongly overexpressed in HT-29 drug-resistant cells. Other drug-resistant cell lines expressed *Reg IV* at a high level, whereas a low expression was noted in sensitive cell lines. Northern blot and real-time PCR analysis showed that *Reg IV* is more strongly expressed in 71% of colorectal tumors (in particular in mucinous carcinomas) than in normal colon tissues. The comparison of *Reg IV* expression with that of other *REG* genes, *Regenerating Iα* or (*Reg Iα*), *Regenerating Iβ* (*Reg Iβ*) and Pancreatitis-associated protein (*PAP*), highlights its predominant expression in colorectal tumors. *Reg IV* mRNA-positive tumor cells display different phenotypes: mucus-secreting, enterocyte-like or undifferentiated. Interestingly, whereas *Reg IV* expression is low in normal colon, its level in normal small intestine is similar to that in some colorectal tumors. In normal tissue, *Reg IV* mRNA-positive cells are mostly enteroendocrine cells and goblet cells. Our results point out the potential role of Reg IV in colorectal tumors and its subsequent interest as a pronostic indicator of tumor survival.
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Key words: *Reg IV; cell survival; drug-resistance; colon cancer cells; colorectal cancer*

Colorectal cancers are highly metastatic and drug-resistant. The genetic alterations associated with colorectal carcinogenesis and the mechanisms involved in their resistance to various drugs have been extensively studied during the last 20 years.[1–5] However, the knowledge accumulated has not led to satisfactory treatments for advanced tumors. A better understanding of the cellular processes leading to general or specific resistance to chemotherapy is required to develop therapy that allows the eradication of drug-resistant tumor cells. Recently, new pathways of resistance have been implicated in the ability of tumor cells to escape drug toxicity. Several pathways of disruption of the apoptosis program have been shown to reduce the chemosensitivity of tumor cells.[6–9] Survival signals provided by cytokines, growth factors, cell-extracellular matrix interactions and cell-cell contacts can modify the intrinsic resistance of tumor cells.[10,11] The occurrence of such mechanisms in tumor cells, defined as cells with a resistance phenotype, could favor the development of specific mechanisms of drug resistance, such as amplification or mutation of the gene encoding the drug-targeted enzyme or drug-transport failure.[1,4,12,13]

The analysis of chemoresistance must also address cellular heterogeneity within a tumor or between tumors. Heterogeneity results from genetic instability of tumor cells[2] and from complex interactions with their microenvironment (growth factors, nutrients, hypoxia, and tumor cell/cell or cell/mesenchyme interactions).[12,14,15] During the last decade, we and other laboratories have observed a relationship between the drug-resistance of tumor cells and the expression of a more differentiated phenotype.[16–21] The few cells of the colon cancer cell line HT-29, mainly constituted of undifferentiated cells, that survive nutrient starvation or exposure to the drugs 5-fluorouracil (5-FU) or methotrexate (MTX) exhibit differentiation characteristics similar to those of normal intestinal cells : cell polarity, presence of apical microvilli and the production of particular proteins including villin, dipeptidylpeptidase IV and mucins.[17,18,20,24] Enterocyte-like HT-29 cells behave like tumor cells exhibiting a resistance phenotype.[13] Similar results have been reported with the enterocytic Caco-2 cell.[21,25] Despite numerous studies of the gene regulation associated with cell differentiation and functions in these models, the mechanisms leading to the resistance phenotype remain unsolved.

Our aim was to identify genes involved in survival pathways developed by drug-resistant tumor cells. Using differential display PCR (DD-PCR)[26] with HT-29 models, we identified a new gene, *Reg IV*, which is strongly overexpressed in HT-29 cells subpopulations that exhibit both drug resistance and differentiation. *Reg IV* has been recently characterized as a new member of the *Reg* multigene family,[27] which includes 3 other genes encoding Reg Iα or Lithostatin A, Reg Iβ or Lithostatin B, and Pancreatitis-associated protein (PAP) (see 28 for review). We show that *Reg IV* expression is increased in most of the colorectal tumors studied in comparison to normal tissues, and in some peritumoral tissues.

MATERIAL AND METHODS

Cell lines and culture conditions

The following cell lines, established from human colorectal adenocarcinomas, were used: HT-29p, Caco-2, LS174T, SW480, HCT-EB, LoVo and LS513.[29,30] The enterocyte-like TC7 and PF11 clones were isolated from Caco-2.[31] The HT-29 clone 19A, provided by C. Laboisse, was obtained from the HT-29 parental cell line (HT-29p) by a sodium butyrate treatment.[32] HT29-Glc$^-$, HT29-FU10^{-6} and HT29-MTX10^{-5} cell populations, and HT29-5M12 and HT29-5M21 clones were selected from HT-29p by nutrient deprivation or long-term treatment with increasing concentrations of 5-FU or MTX as previously described.[13,17,20,24] All cell lines were cultured as previously described.[17,19,20,29] with modifications for Caco-2 and its subclones.[31] Experiments were

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done in 25 cm^2 or 75 cm^2 T-flasks (Corning Glass Works, Corning, NY) at 37°C under a 10%CO_2/90% air atmosphere. Cells were seeded at different densities to reach confluency after the same time of culture: 0.6×10^4 cells per cm^2 for Caco-2 and subclones, 0.8×10^4 for LoVo and 2×10^4 for all other cell lines. The medium was changed daily. The long-term treatment of cell lines with 5-FU (Sigma Chemical Co., St. Louis, MO) or MTX (generously provided by Rhone-Poulenc-Rorer) was administrated to exponential cells at the indicated concentrations, as previously described for HT-29.[17,20] The drug-resistance capacities of these cell lines have been detailed elsewhere.[25]

Tissues

For *in situ* hybridization, specimens of normal adult jejunum and sigmoid, obtained from patients without any evidence of neoplastic disease and samples of colorectal tumors, were immediately immersed in fresh 10% phosphate-buffered formalin and then embedded in paraffin. Four micrometer-thick sections were cut, mounted on gelatin-covered slides and stored at 4°C until use. Serial sections were systematically stained with hematoxylin-eosin-safran or Alcian blue (pH 2.5) for histological analysis.

For Northern blot and real-time PCR analysis, human gastrointestinal tissues (jejunum, colon and pancreas) were obtained from irreversibly brain-damaged organ donors, according to protocols approved by the National Ethical Committee, and are referred to as Normal. Tumor samples were obtained from 35 resected primary colorectal carcinomas. Patients had received no adjuvant chemotherapy at the time at which these samples were collected. In 13 cases, non tumoral samples of peri-tumoral mucosa were obtained. Three hepatic metastases with the corresponding nontumoral tissue were also studied. Tumor samples were supplied by Prof. Capron and Dr. Gruffaz (Hôpital Beclert, Clamart, France), Prof. Reynes and Dr. Sebagh (Hôpital Paul Brousse, Villejuif, France), Prof. Bouillot (Hôtel-Dieu, Paris, France) and Dr. de Mestier (Hôpital, Paris, France). All specimens for RNA assay were snap-frozen in liquid nitrogen and stored in liquid nitrogen until analysis.

Differential display and isolation of full-length Reg IV *cDNA*

Differential display was performed as described[26] with an RNAimage kit (GenHunter, Nashville, TN) according to the manufacturer's instructions. Total RNA purification from exponentially growing cells (HT-29p, HT29-Glc$^-$, HT29-5M12 and HT29-FU10^{-6})[33] included a treatment with DNase I (Boehringer-Mannheim, Indianapolis, IN). The anchor and arbitrary primers that led to detection of Reg IV cDNA (386 bp) were 5′-AAGCTTTTTTTTTTTA-3′ and 5′-AAGCTTAGAGGCA-3′. To isolate a larger cDNA fragment (804 bp), a λZap express cDNA library from stationary phase TC7 cells was screened by PCR using proofreading thermostable DNA polymerase (ClonTech, Palo Alto, CA) and a 5′ T3 primer and 3′ reverse primer (5′-GAGCTAGAAGCCACTACTG-3′), corresponding to the 5′ part of the 386 bp insert. Finally, the extreme 5′ end was isolated from a Marathon-Ready cDNA library prepared from human normal intestine (Clontech) by 5′RACE-PCR using the 5′ AP1 primer and an internal primer (5′-CAGGAAGTGTTGGCGCTTGT-3′).

Northern blot analysis

Total RNA was extracted from cells (exponentially growing or stationary phase) or from tissue samples (normal, peritumoral or tumoral) using guanidium isothiocyanate and centrifugation through a CsCl gradient as described.[33] RNA samples (20 μg) were fractionated by electrophoresis, and Northern blotting was done as previously reported[13] using the 804 bp fragment of Reg IV as the probe. A 3′ end-labeled oligonucleotide hybridizing to 18S rRNA was used for normalization: the 18S rRNA was used as internal control to quantify *Reg IV* expression with the Gel analyst 3.0 program.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from 10 30 μm-thick cryostat sections using RNA instapure (Eurogentec, Seraing, Belgium) according to the manufacter's protocol. Reverse-transcription was carried out with 1 μg of RNA, 8 μM of hexamer primer pool, 250 μM dNTP, 30 U RNaseOUT, 10 mM DTT, 300 U MMLV Reverse Transcriptase and RT buffer ×1 in a total volume of 30 μl. After incubation at 42°C for 1 hr, cDNA was stored at −20°C or immediately used for real-time PCR. *REG* mRNA was determined semi-quantitatively with a LightCycler System (Roche Molecular Biochemicals, Indianapolis, IN). PCR reactions were performed using 2.5 μl of a 1:25 dilution of each cDNA and 7.5 μl reaction volume containing 1× SYBR Green I master mix, 0.4 μM (*Reg IV* and *L19*) or 0.8 μM (*Reg Iα*, *Reg Iβ* and *PAP*) of each specific primer, and 3 mM $MgCl_2$ (except for *Reg Iβ*:4 mM). The specific primers used for human *Reg Iα*, *Reg Iβ* and *PAP* were as previously described.[34] 5′-GTCTGATGCCGAGCTCGAGTG-3′ and 5′-CAGGAAGTGTTGGCGCTTGT-3′ were used for *Reg IV* analysis. As an internal control for the RNA extraction and reverse-transcription, ribosomal protein *L19*-specific primers were used.[35] The conditions for the amplification were an initial denaturation at 95°C for 8 min, followed by 10 sec at 95°C, 10 sec at 60°C (*L19*), 62°C (*Reg Iα*, *Reg Iβ* and *PAP*) or 64°C (*Reg IV*) and 10 sec at 72°C for 45 cycles. Results are expressed as the ratio of the level of *REG* mRNA to that of *L19* mRNA in arbitrary units. To estimate the level of expression of each *REG* gene in colorectal tumors, these results were compared to a reference value: the average of the values obtained for 4 normal colon samples.

Immunohistochemistry

Prior to the hybridization procedure, some intestinal sections were incubated for 12 hr at 4°C in rabbit antiserum specific for Chromogranin A (Dako, Glostrup, Denmark) diluted 1:100 in Tris-buffered saline (TBS). After washing, sections were incubated for 1.5 hr in biotinylated goat antirabbit serum (1:200 in TBS, Caltag Laboratories, Burlingame, CA), and then in peroxidase-conjugated avidin (Vector Laboratories, Burlingame, CA). Sections were processed for the DAB reaction by standard procedures. The specificity of the IHC reaction was verified by replacement of the primary antibody with normal rabbit serum, in which case there was no immunoreaction.

RNA probes and in situ *hybridization*

The 804 bp fragment of Reg IV was inserted into the vector pCR3.1 using the TA cloning system (Invitrogen, La Jolla, CA) according to the manufacturer's instructions. pCR3.1 plasmids that contained the sense or antisense 804 bp fragment were linearized with Pst I and used to synthesize sense and antisense human *Reg IV* probes using ^{35}S-CTP (specific activity above 1,000 Ci/mmol; Amersham, UK) and T7 RNA polymerase.

ISH was performed by the standard procedure of Mitchell *et al.*[36] Briefly, after proteinase K permeabilization, sections were hybridized with riboprobe-hybridization buffer mix, which contained the ^{35}S-labeled Reg IV cRNA probe (40,000 cpm/μl). Overnight hybridization at 55°C was followed by RNase treatment and a series of stringent washes, including a high-stringency wash at 60°C. Hybridized slides were dehydrated in 70% and 100% ethanol in ammonium acetate and dipped in NTB2 emulsion (Kodak, France). After 20 days exposure, slides were developed.

The controls for specificity of the probe were checked: incubation of the sections with ^{35}S-labeled sense probe, pretreatment with ribonuclease and co-incubation with a 100-fold excess of unlabeled antisense probes all gave no specific labeling.

RESULTS

Differential gene expression between drug-resistant and sensitive colon tumor cells

To identify genes potentially involved in the resistance phenotype of tumor cells, we compared the mRNAs present in drug-resistant and differentiated HT-29 subpopulations with those of drug-sensitive and undifferentiated parental cell line HT-29p. The resistant subpopulations were selected from exponentially growing

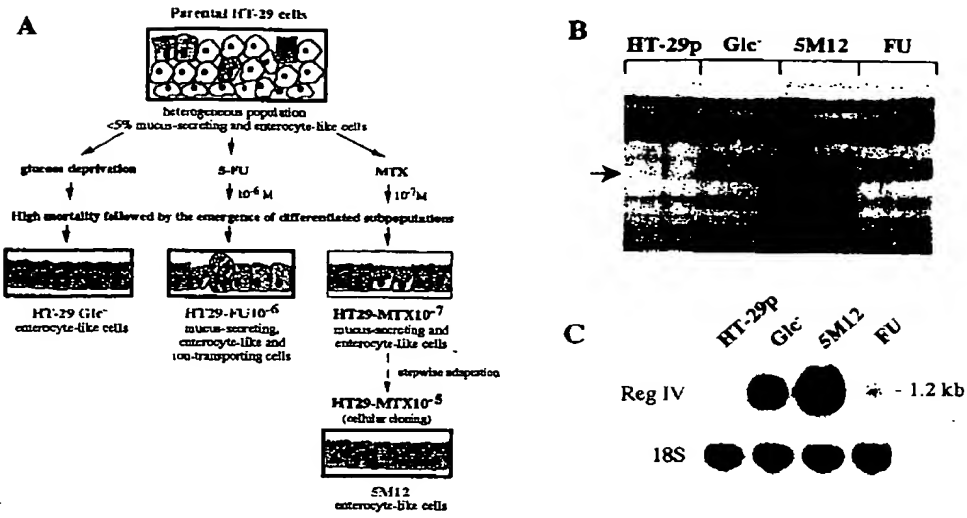

FIGURE 1 – Identification of *Reg IV*, a gene differentially expressed in colon cancer cells sensitive (HT-29p) or resistant to drugs used in chemotherapy (HT29-Glc⁻, HT29-FU10^{-6} and HT29-5M12). (*a*) Origin and characteristics of the 4 cell populations used for the differential display analysis. HT29-Glc^{-} (Glc^{-}). HT29-5M12 (5M12) and HT29-FU10^{-6} (FU) were selected from the HT-29p cell line by nutrient deprivation or by long-term treatment with MTX or 5-FU as previously reported.[13,20,24] The 2 first cell lines are resistant to both 5-FU and MTX (13). (*b*) Differential display of the mRNAs isolated from the sensitive and undifferentiated HT-29p cells and from the 3 subpopulations of resistant and differentiated cells (Glc^{-}, 5M12 and FU). Arrow indicates Reg IV cDNA fragment. (*c*) This ^{32}P labeled cDNA fragment hybridized to a 1.2 kb mRNA which is up-regulated in the 3 HT-29 subpopulations. The 18S rRNA is shown as a loading control.

TABLE I – DIFFERENTIAL DISPLAY EXPERIMENT: CHARACTERIZATION OF THE 13 SELECTED CLONES

cDNA clone	Homology with (AC)[1]	Protein	mRNA abundance in resistant cells
13.1	U30521	P311	INCREASED in the 3 populations
125.3	AY007243	Reg IV	
28.2	AL356494	Unknown	INCREASED in HT29-Glc^{-} and 5M12 cells
28.7	NM014908	KIAA1094 protein	
44.9	X93334	cox II[2]	
15.1	XM008756	P84	INCREASED in HT29-FU cells
2.5	M29551	Calcineurin A2	DECREASED in HT29-Glc^{-} and 5M12 cells
25.8	X07549	Cathepsine H	
28.6	AF026293	CCT-β[2]	
33.3	BC009677	ATPase inhibitor precursor	
38.4	D89870	Antizyme	
39.10	XM006559	PHLDA1[2]	
45.4	XM057840	Unknown	

[1]AC, EMBL/GenBank accession number–[2] cox II, Cytochrome C oxydase subunit II; CCT-β, Chaperonin containing-complex polypeptide 1 beta subunit; and PHLDA1, Pleckstrin homology-like domain, family A number 1.

HT-29p cells as shown in Figure 1*a*. We used 3 drug-resistant cell populations for the screening, HT29-Glc^{-}, HT29-5M12 and HT29-FU10^{-6}, to identify genes involved in a resistance phenotype expressed by more than 1 tumor cell line.

Differential display-PCR was performed using total RNAs extracted from the 4 cell cultures at 40% confluency. After using 25% of the available primer sets, we selected 62 bands, in sequencing gels, displaying differential expression. Most differed between HT-29p and enterocyte-like HT29-Glc^{-} and HT29-5M12 cells, although some differed between HT-29p and the 3 HT-29 subpopulations (data not shown). Thirty-four differential expressions were confirmed by Northern blot. Thirteen genes were identified by sequencing (Table I), 2 encoding proteins to which no function has been attributed. Interestingly, some of the genes encode proteins involved in general biological processes such as signaling pathways, protein addressing or degradation, polyamine metabolism, cell cycle and apoptosis. One of the bands in the sequencing gels was very intense in drug-resistant cell lines HT29-Glc^{-} and HT29-5M12, weaker in resistant cell line HT29-FU10^{-6} and barely detectable in sensitive HT-29p cells (Fig. 1*b*, arrow).

The corresponding cDNA hybridized in Northern blots with an mRNA of about 1.2 kb (Fig. 1*c*). As expected from the DD-PCR screening, this mRNA was very abundant in both enterocyte-like cells, HT29-5M12 (115-fold the level in sensitive HT-29p cells) and HT29-Glc$^-$ (70-fold), and abundant in HT29-FU10^{-6} cells (23-fold) (Fig. 1*c*). The entire cDNA was cloned and sequenced (1,142 nucleotides; accession number: AF345934) and was 98.4% identical with a cDNA in data banks corresponding to the regenerating gene type IV mRNA (*Reg IV*) (accession number: AY007243), identified in inflammatory bowel and described while this work was being completed.[27]

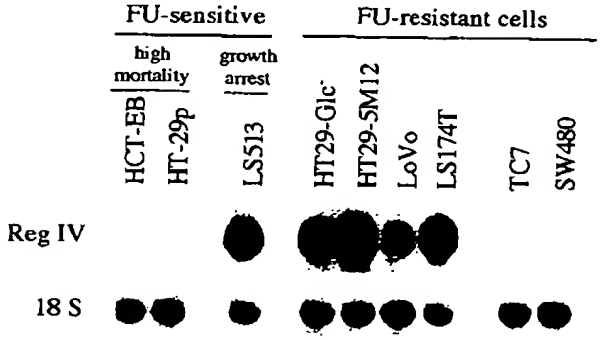

FIGURE 2 – Northern blot analysis of *Reg IV* gene expression in 5-FU sensitive (HT-29, LS513 and HCT-EB) or resistant (HT29-Glc$^-$, HT29-5M12, LS174T, TC7, SW480 and LoVo) cell lines. Note the high level of *Reg IV* mRNA in the sensitive LS513 cells that survive but do not grow in presence of 5-FU. Only HCT-EB and HT-29p of these cell lines displayed a high mortality during a long-term treatment with 0.1 μM of methotrexate. RNAs were prepared from exponentially growing cells.

Reg IV *mRNA is overexpressed in several drug-resistant cell lines*

We identified Reg IV in HT-29 cells that exhibited a drug-resistant phenotype. To further assess a potential association of Reg IV overexpression with drug resistance, we evaluated the abundance of *Reg IV* mRNA in various human colorectal cell lines that behave differently upon a long-term treatment with 5-FU. Cell lines (HT29-Glc$^-$, HT29-5M12, LS174T, TC7, SW480 and LoVo) which are able to proliferate in the presence of the drug and classified as resistant,[13,25] were tested. Four resistant cell lines (HT29-Glc$^-$, HT29-5M12, LS174T and LoVo) contained high levels of *Reg IV* mRNA (Fig. 2); TC7 contained very little and SW480 undetectable amounts of *Reg IV* (Fig. 2), thus underlining the heterogeneity of drug-resistance mechanisms of colon tumor cells. Very little or no *Reg IV* mRNA was present in the cell lines HT-29p and HCT-EB, which are drug-sensitive and display a high mortality during 5-FU treatment.[13,25] Interestingly, LS513 cells, which display their drug-sensitivity by growth arrest but low mortality upon a 5-FU treatment[25] contained abundant *Reg IV* mRNA.

The Reg IV *gene is overexpressed in colorectal tumors*

We estimated, by Northern blot, the *Reg IV* mRNA levels in several peritumoral and tumoral colon mucosa specimens from the same patients and in normal colon mucosa. The 5 normal colon specimens contained less *Reg IV* mRNA than most of the tumors, although the levels of *Reg IV* mRNA differed between individuals (Fig. 3*a*). We observed that 77% (10/13) of colorectal tumors displayed a higher level of *Reg IV* mRNA than various normal colon mucosa specimens (Fig. 3*a,b*). In 4 cases, *Reg IV* mRNA

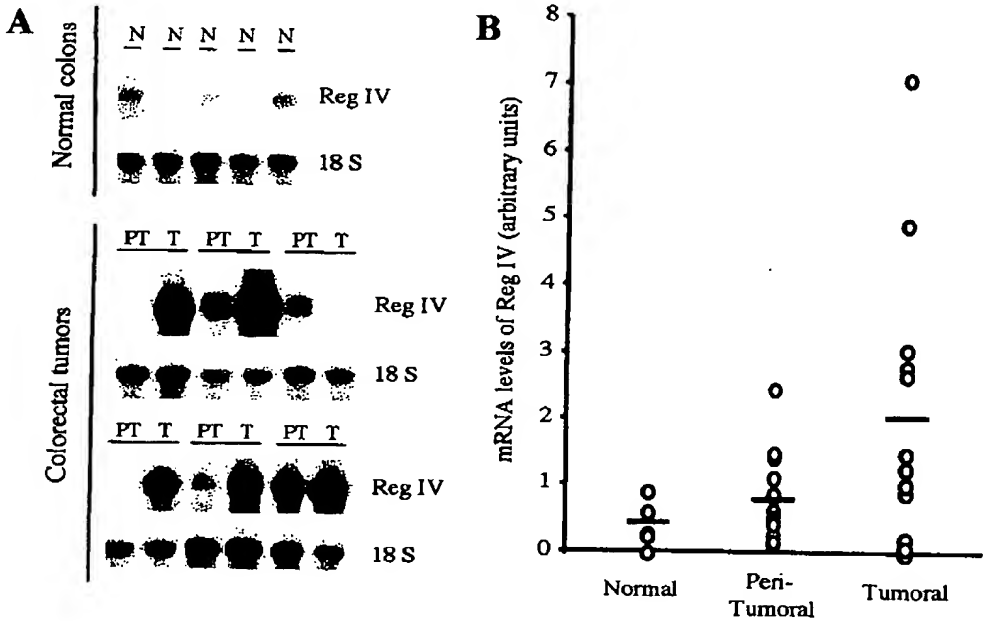

FIGURE 3 – *Reg IV* mRNA in normal, peritumoral and tumoral colon. (*a*) Northern blot analysis of total RNAs isolated from tumoral or peritumoral tissues and from normal colon. RNAs were extracted from 5 normal colons (N) and from 13 colorectal tumors (T) and also from their corresponding peri-tumoral mucosa (PT). Six representative pairs of samples are shown. (*b*) Graphic representation of *Reg IV* mRNA levels in normal, peritumoral and tumoral tissues. The *Reg IV* mRNA was quantified with respect to the 18S rRNA detected by hybridization on the same membrane, using the Gel analyst 3.0 software. The horizontal bar indicates the average values for each category.

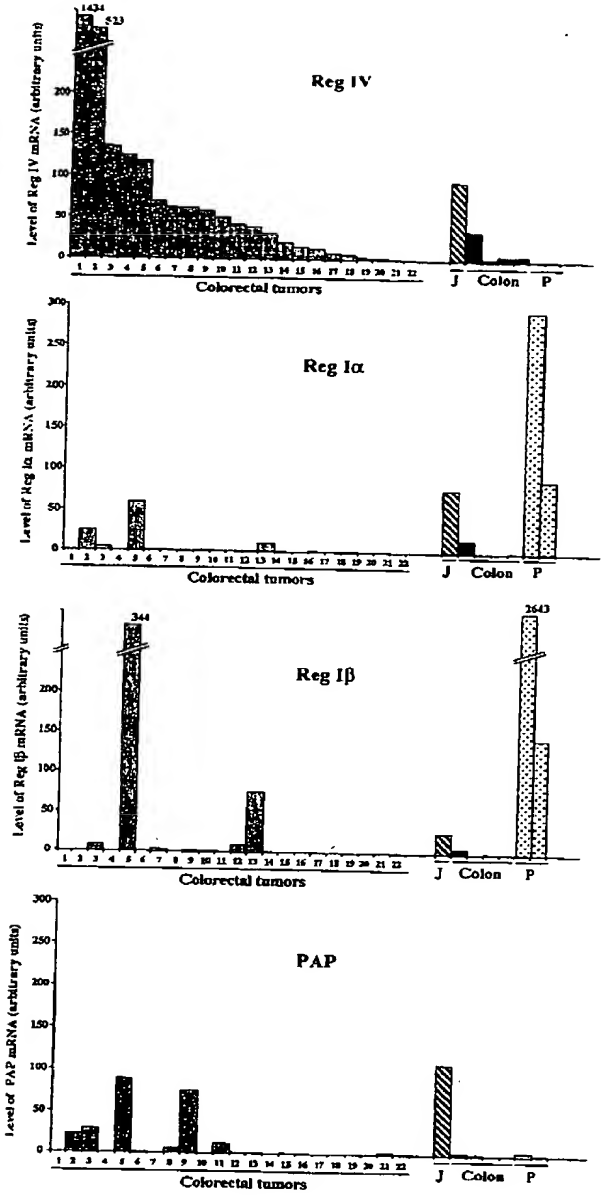

FIGURE 4 – Comparison of *Reg Iα*, *Reg Iβ*, *PAP* and *Reg IV* transcript levels in colorectal tumors by real time-PCR for 22 tumor samples and normal tissues. Jejunum (J), colon and pancreas (P) samples were used as controls for *REG* expression. To quantify the level of expression of each *REG* gene, the value for the *Reg* mRNA is expressed as a ratio to that of *L19* mRNA. The reference level for each gene in normal colon represents the average of *Reg* mRNA levels in the 4 colons analyzed.

was clearly elevated in peri-tumoral mucosa (Fig. 3*a*, representative samples). In 3 samples, the peri-tumoral mucosa had higher levels of *Reg IV* mRNA than the corresponding tumor (Fig. 3*a*). The average level of *Reg IV* mRNA in the 3 tissue classes is shown in Figure 3*b*. The average level of *Reg IV* mRNA is clearly higher

TABLE II – *REG IV* EXPRESSION ACCORDING TO CLINICAL FEATURES

Variable	Patients	Overexpression of *Reg IV* in comparison to normal colon
Total number studied	35	25/35 (71.4%)
Sex		
Male	16/35	9/16 (56.2%)
Female	19/35	16/19 (84.2%)
Tumor site		
Right colon	11/35	9/11 (81.8%)
Left colon	17/35	12/17 (70.6%)
Rectum	7/35	4/7 (57.1%)
Tumor stage		
I ($T_{1-2}N_0M_0$)	5/35	3/5 (60%)
II ($T_{3-4}N_0M_0$)	12/35	8/12 (66.6%)
III ($T_{1-4}N_{1-2}M_0$)	12/35	10/12 (83.3%)
IV ($T_{1-4}N_{0-2}M_1$)	6/35	4/6 (66.6%)

in the tumoral samples than that in the healthy tissues. Three liver metastasis samples associated with a primary colonic tumor were compared to healthy liver tissue from the same individuals. *Reg IV* mRNA was not found in normal liver samples, as previously reported[27] but small amounts were detected in 2 of the 3 liver metastasis samples (data not shown).

Comparison of Reg IV *gene expression to that of the other* REG *genes in colon carcinomas*

Twenty-two tumor samples were analyzed semi quantitatively by real time PCR of the *REG* genes. *Reg Iα*, *Reg Iβ*, *PAP* and *Reg IV*. Normal small intestine (jejunum), colon and pancreas were used as controls of *REG* gene expression (Fig. 4). Consistent with previous reports,[37–39] we observed a stronger expression of *Reg Iβ* and to a lesser extent of *Reg Iα* in pancreas than in jejunum and colon. As expected,[40,41] we found that *PAP* was more weakly expressed in pancreas and colon than in jejunum. We also found that *Reg IV* expression was higher in normal small intestine than in normal colon; and in normal pancreas. *Reg IV* transcripts were barely detectable (Fig. 4).

The expression profiles of the 4 *REG* genes were different in colorectal tumors (Fig. 4). As observed with the 13 tumor samples described above, *Reg IV* was more strongly expressed in most colorectal tumors (68.2%) than in normal colons (Fig. 4). The level of *Reg IV* mRNA varies within a range of 4 orders of magnitude between the different tumors. In only 6 samples of 22, the expression was comparable to that of the 4 normal colon samples. Unlike *Reg IV*, the other *REG* mRNAs were present at very low or undetectable levels in most samples. Only 13.6% of the 22 tumors exhibited a higher expression of *Reg Iα* than that in normal colons : the corresponding values were 22.7% and 31.8% for *Reg Iβ* and *PAP*, respectively. In only 1 case (tumor number 5) the 4 *REG* genes were concomitantly up-regulated. It is noticeable that the 9 colonic cell lines (Fig. 2) exhibited the same pattern as the tumors: *Reg IV* was the most abundantly expressed gene whereas the expression of the 3 other genes tested was barely or not detectable (data not shown).

Detection of Reg IV *mRNA in tumoral cells*

Using all 35 colorectal tumors, we searched for a possible link between the level of *Reg IV* expression and clinical features. In our study, no significant relationship was found between the presence of *Reg IV* transcripts or the increase of *Reg IV* expression, and the TNM state of the tumors or their localization (Table II). However, the histological analysis of the tumors revealed that the most of colorectal tumors overexpressing *Reg IV* were mucinous tumors or, in some cases, neuro-endocrine tumors. In the absence of antibodies specific for Reg IV, we could not study the protein localization or regulation. We analyzed *Reg IV* expression in the colorectal tumors by *in situ* hybridization (Fig. 5*a–c*); staining of serial sections with alcian blue visualized the presence of mucus (Fig. 5*e–g*). The pattern of *Reg IV* expression differed between

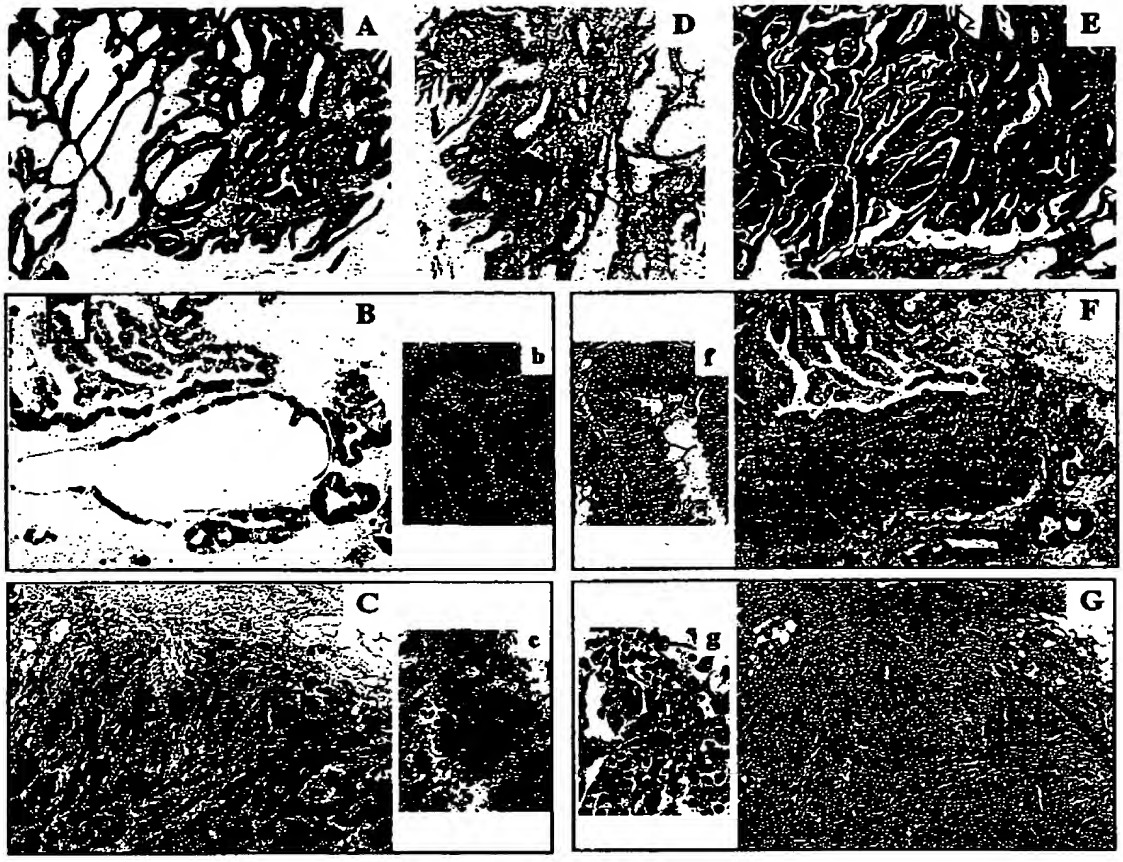

FIGURE 5 – Reg IV *in situ* hybridization and Alcian blue staining of colorectal tumors. (*a–c*) *Reg IV* mRNA-positive cells were present at variable densities in tumors. Note the absence of signal in the stroma. (*d*) No labeled cells were observed with the Reg IV sense probe. (e–g) : the presence of mucus was detected by alcian blue staining in 2 tumors (*e* and *f*). Note that in the undifferentiated tumor (*g*), only the stroma cells are weakly stained with alcian blue. The enlargements of tumor sections (inserts b, c, f, g) show that many Reg IV-positive cells were mucus-secreting cells but some were not (*b, f*), and that many non-mucus-secreting cells (*g*) were labeled with Reg IV (*c*). All sections were hybridized with the same preparation of ^{35}S-labeled probe and exposed for the same time. Methyl green pyronin counterstain for ISH sections. Original magnifications: (*a* to *g*) ×25, (inserts *b, c, f, g*)×100.

tumors. The signal was clearly localized in the epithelial-derived tumor cells and not in the stromal cells (Fig. 5*a–c*). Mucinous tumors (Fig. 5*e,f*, and insert f) found to be Reg IV-positive by RT-PCR gave strong signals with the antisense probe (Fig. 5*a,b*, and insert b) but not with the sense probe (Fig. 5*d*). Interestingly, sections of alcian blue-negative tumor (Fig. 5*g*, and insert g) were also positively labeled with the Reg IV antisense probe (Fig. 5*c*, and insert c), indicating that *Reg IV* expression is not restricted to mucus-secreting cells.

Accordingly, the tumor colon cell lines displaying a mucus-secreting phenotype (HT29-MTX10^{-5} and HT29-5M21) had higher levels of *Reg IV* mRNA than those in the undifferentiated HT-29p cell line (Fig. 6). Some cell lines are very similar to small intestine (Caco-2 and its 2 clones, PF11 and TC7)[29,31] or colon (HT29-5M12, HT29-5F7 and HT29-19A) enterocytes.[13,19] Interestingly, *Reg IV* expression differed considerably between these cells: HT29-5M12 and HT29-5F7 cells contained more *Reg IV* mRNA than Caco-2, TC7 and PF11 cells (Fig. 6). The abundance of *Reg IV* mRNA in Caco-2, TC7 and PF11 cells was comparable to that in undifferentiated HT-29p cells. We also observed that in enterocyte-like HT29-19A cells and goblet HT29-MTX10-5 and

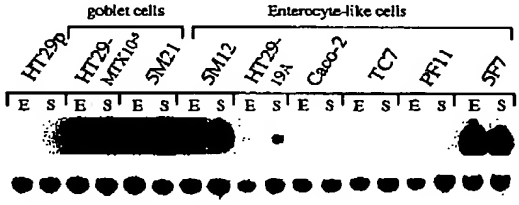

FIGURE 6 – Northern blot analysis of *Reg IV* gene expression in colon tumor cell lines displaying an enterocyte-like or a goblet cell phenotype. RNAs were prepared from exponentially growing (E) or stationary phase (S) cultures. 18S rRNA was used as loading control.

HT29-5M21 cells the levels of *Reg IV* mRNA were higher in nondividing (stationary growth phase) than in dividing (exponentially growing) cells (Fig. 6). No such difference was seen in cells displaying a higher level of *Reg IV* expression during the exponential phase (HT29-5M12 and HT29-5F7). Thus, studies with cell lines confirmed that *Reg IV* expression is not restricted to mucus-

secreting cells but that enterocyte-like cells also express the *Reg IV* gene.

Identification of the Reg IV *mRNA-expressing cells in normal intestine*

Reg IV was expressed at a higher level in human small intestine (jejunum) than in normal colon (Fig. 4). We attempted to identify the cell types in small intestine and colon producing *Reg IV* mRNA, by *in situ* hybridization and specific staining. *Reg IV* mRNA-positive cells were found in small intestine in accordance with mRNA analysis. Sparse along the intestinal epithelium, some cells displayed a positive signal, both in crypts and villi (Fig. 7*a*).

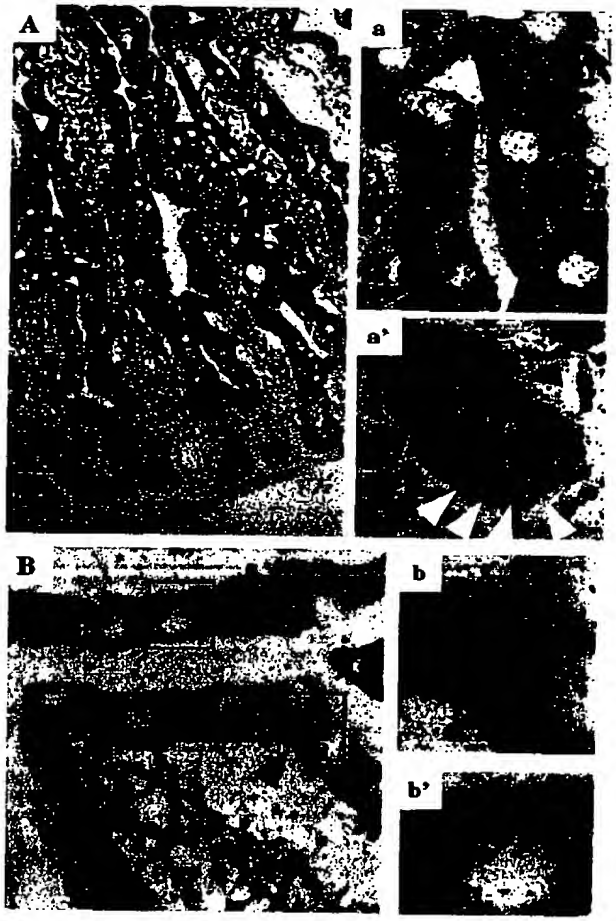

FIGURE 7 – *In situ* hybridization for *Reg IV* mRNAs in normal jejunum. (*a*) The signal is strong in some cells of the crypts and villous. Insert a: Note the clear labeling of the perinuclear region of goblet cells (black arrows) and also in an unidentified cell (black arrowhead). Insert a': The Paneth cells are negative (white arrowheads). Note the strong labeling in 2 unidentified cells of the crypt (black arrowheads). Sections were exposed for 20 days. (*b*) The entero-endocrine cells, stained with a polyclonal antibody specific for chromogranin A, also present a strong signal with the Reg IV antisense probe. Insert b: double positivity of entero-endocrine cells. Insert b': *Reg IV* mRNA-positive goblet cell. Note the difference of labeling with the enteroendocrine cell of insert b. Sections were exposed for 13 days. Methyl green pyronin counterstain. Original magnifications: (*a*) ×100, (inserts a, a') ×200. (*b*) ×200. (inserts b, b') ×400.

Reg IV mRNA was observed at the basal perinuclear zone of goblet cells, which are easily identifiable by their apical mucus vacuoles (see insert a Fig. 7*a*). Entero-endocrine cells, identified in the villi and crypts by their chromogranin A immunoreactivity, exhibited a strong signal (Fig. 7*b*, insert b). Neither all the goblet cells nor all the entero-endocrine cells presented the same level of *Reg IV* expression. The enterocyte cells, that constitute 80% of the villous epithelium, and the Paneth cells, which are located in the bottom of the intestinal crypts, were negative for *Reg IV* mRNA (Fig. 7*a* and insert a'). In normal colon, there were few *Reg IV* mRNA-expressing cells in Lieberkühn glands (data not shown). This observation confirmed the low levels of *Reg IV* transcripts detected in the normal colon by northern blot and real-time PCR (Figs. 3 and 4).

DISCUSSION

Colon tumors display cell heterogeneity, and they are associated with a poor prognosis, which results from the multiplicity of drug resistance mechanisms that they develop. Well-differentiated tumors grow slower than those that are undifferentiated but are less sensitive to chemotherapy. Accordingly, long-term treatment of drug-sensitive and undifferentiated HT-29 cells results in the selection of drug-resistant subpopulations that express an enterocytic-like or goblet cell phenotype.[13,17,18,20] To identify the survival pathway(s) developed by colon cancer cells, we used DD-PCR screening to compare HT-29 drug-resistant and -sensitive subpopulations. We thereby identified *Reg IV* as a gene overexpressed in drug-resistant HT-29 cells. Interestingly, 4 of 6 cell lines described as resistant to long-term treatment with 5-FU[13] also presented a high level of *Reg IV* mRNA. However, the TC7 and SW480 cell lines are drug-resistant but have low levels of *Reg IV* mRNA. As demonstrated in other systems,[1,4,12] there are diverse mechanisms of resistance to drug toxicity and, at this stage of our work, we can not establish a direct relationship between chemoresistance and *Reg IV* expression. We are currently studying the time course of *Reg IV* up-regulation during the long-term treatment with 5-FU and also with MTX.

Up-regulation of *Reg IV* has been reported in Crohn's disease and ulcerative colitis by Hartupee *et al.*, following high-throughput sequence analysis of a large bowel inflammatory disease library.[27] We report here that *Reg IV* is strongly expressed in most colorectal carcinomas, whereas *Reg IV* mRNA levels were low or undetectable in normal colon. *Reg IV* has been classified in the *REG* gene family, 2 members of which, *Reg Iα* and *PAP*, are also up-regulated in pathological tissues: pancreatitis, colon and liver carcinomas.[28,34,40,42,43] *REG* genes are expressed at comparable levels in normal colon, but *Reg IV* is the only one that presents major modifications of its expression in colorectal tumors and in various colon tumor cell lines. Moreover, peri-tumoral mucosa also exhibited stronger expression of *Reg IV* than normal colon mucosa, suggesting that deregulation of Reg IV could be an early event preceding the apparition of histological markers of tumor in the peri-tumoral mucosa. As reported for *Reg Iα* and *PAP*,[34,43] there is no clear relationship between *Reg IV* gene expression and the localization or stage of the colorectal tumors studied. Macadam *et al.* have recently shown that, in patients with non-metastatic colorectal tumor, the co-expression of Reg Iα and *PAP* had a highly significant adverse effect on survival.[43] Clinical follow-up of a large number of patients over several years is needed to determine the relationship between Reg IV and survival.

In our study of colon cancers, we found that the tumors expressing Reg IV most strongly were those displaying a mucinous phenotype. However, non mucus-secreting tumor cells were also able to express Reg IV. We confirmed this result using various cell lines. Among differentiated cells, enterocyte-like cells contained high levels of *Reg IV* mRNA (HT29-5M12, HT29-5F7 and HT29-19A). Strong *Reg IV* expression was also detected in undifferentiated cell line SW480 (see Fig. 2). Strikingly, *Reg IV* is expressed in normal adult small intestine at a higher level than in normal

colon, and indeed at a level similar to that observed in various colorectal tumors. In normal adult small intestine, many entero-endocrine and goblet cells of the crypt-villus epithelium strongly expressed *Reg IV*, with the entero-endocrine cells being the more intensely labeled in ISH experiments. Unlike *Reg IV*, *PAP* and *Reg Iα* are mostly expressed in Paneth cells, a type of resident epithelial cells in small intestine crypts.[38,40,41]

Expression of *Reg IV* in different cell types in normal intestine and colon and in colon tumors raises questions about its function. Various physiological functions have been ascribed to REG proteins. Their expression has been associated with proliferation and regeneration,[28,37,44] cell survival,[45] cell adhesion [40] and resistance to apoptosis.[46,47] From our results, we can not conclude that REG IV acts as a mitogen or growth factor as proposed by Hartupee *et al.*, based on its overexpression in Crohn's disease or ulcerative colitis.[27] Even though the *Reg IV* gene is expressed by some cells of the intestinal crypt, which comprises the proliferating cells of the intestinal epithelium, *Reg IV* mRNA is also abundant in the non dividing cells of the intestinal villus. The *Reg IV* gene is similarly expressed in growing and in resting colorectal cell lines, suggesting that REG IV does not interfere directly with proliferation mechanisms. Furthermore, LS513 cells that present a growth arrest during 5-FU treatment[25] contained abundant *Reg IV* mRNA (Fig. 2). Besides, PAP and REG Iα are secreted proteins and have been recently shown to act as growth factors.[28,45] Analyses of PAP in mouse embryos have demonstrated its early expression in the nervous system, pancreas and small intestine[42,45] and indicated that it is a signaling intermediate in a survival pathway of motoneurons.[45] The analysis of the amino acid sequence of Reg IV suggests that it may be secreted (our study and 27), and REG IV protein has been found in the culture medium of HT29-5M12 cells by 2-dimensional electrophoresis and mass spectrometry (Delphine Delacour and Guillemette Huet, personal communication). Therefore, as a secreted protein, REG IV may act on the neighboring cells in normal or tumoral tissues as described for REG Iα or PAP[28,45] Finally, it may have a role in resistance to apoptosis, as suggested by the high cell mortality during 5-FU exposure of HCT-EB and HT-29p cell lines[25] in which the level of *Reg IV* mRNA is very low. Few cells of the HT-29p cell line are able to escape drug-treatment or nutrient deprivation (see Fig. 1 and 17, 20, 24), and the majority of them express high levels of *Reg IV* following these stresses.

Our data concerning *Reg IV* expression, as assessed by studying the mRNA level, are in accordance with the suggestion of Macadam and collaborators[43] that colorectal tumor cells expressing REG proteins have an advantage in terms of survival. Our results suggest that Reg IV-positive cells in colorectal tumors may also have a selective advantage during drug-treatment. Further studies are necessary to establish a direct relationship between cell survival and *Reg IV* expression, and to evaluate REG IV protein as a new marker for the detection of colon cancers and as a pronostic indicator.

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